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"Where, O Death, Is Thy Sting?" A Brief Review of Apoptosis Biology

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Abstract Apoptosis was a term introduced in 1972 to distinguish a mode of cell death with characteristic morphology and apparently regulated, endogenously driven mechanisms. The effector processes responsible for apoptosis are now mostly well known, involving activation of caspases and Bcl2 family members in response to a wide variety of physiological and injury-induced signals. The factors that lead of the decision to activate apoptosis as opposed to adaptive responses to such signals (e.g. autophagy, cycle arrest, protein synthesis shutoff) are less well understood, but the intranuclear Promyelocytic Leukaemia Body (PML body) may create a local microenvironment in which the audit of DNA damage may occur, informed by the extent of the damage, the adequacy of its repair and other aspects of cell status.

Keywords Cell death · Apoptosis biology · DNA

Introduction

Over the past two or three decades, a clear and seemingly comprehensive picture of the biology of apoptosis has emerged. Originally identified through its characteristic cytological morphology [1], this mode of death is now known to result from activation of a common mechanism relevant in both physiological and pathological circumstances [2, 3]. At the heart of this mechanism lie two families of proteins, the *caspases* and members of the *Bcl2 extended family*. Caspases are a unique and closely related set of proteases, so called because of the cysteine at their active site, and the tightly defined four-amino-acid motif (including aspartate at positions 1 and 4) at their target site. The Bcl2 family is so called because of the relationship of its members to the B-cell lymphoma oncogene whose discovery led eventually to the identification of most of the other family members, but at the molecular level this family is remarkably diverse.

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Caspase Activation Underlies Most of the Phenotype of Apoptosis

The caspases form a cascade in which initiator caspases are activated by lethal stimuli arising either at the cell membrane as a result of cytokine–receptor binding, or within the cell, in relation to internally determined signals, often generated in the micro-environment of particular organelles. Thus, caspases 8 and 10 are activated when specific extracellular ligands of the tumour necrosis factor family bind to their receptors (the extrinsic apoptosis pathway), whilst caspase 9 is activated at the mitochondrial membrane (the intrinsic pathway). These initiator caspases activate (by cleavage at their specific target sites) a set of effector caspases, notably caspases 3, 6 and 7, which then synchronously cleave proteins in many cell compartments. This cleavage event is responsible for most of the morphological changes by which apoptosis was originally identified. Thus, the violent blebbing of apoptotic cells is attributable to activation, by caspase cleavage, of the rho-kinase isoform ROCK-1 [4]. Caspase substrates also include cytoskeleton proteins [5] and the focal adhesion kinase (FAK) [6], whose cleavage accounts for the loss of substratum contact and the loss and rounding up of the

dying cell. Some caspase substrates form part of complex pathways with several interacting members. One striking example is the cytoplasmic chaperone inhibitor of caspase-activated DNAse (ICAD), whose cleavage releases a nuclease from its anchor within the cytoplasm and permits its unfolding to reveal a nuclear localisation signal [7]. The unfolded active nuclease thus arrives within the nucleus where it is responsible for the cleavage and much of the condensation of nuclear DNA. Caspases may also be responsible for the release of nucleotides from apoptotic cells that serve as homing signals for the macrophages that ultimately engulf them [8]. Interestingly, the 4-amino-acid motifs that characterise caspase cleavage sites in scores if not hundreds of proteins [9] appear to be conserved between species as widely divergent as Drosophila, Xenopus and mammals [10, 11]. Thus, the activation of caspases is central to the synchronised molecular events that occur in apoptosis, although it must be said that it is still unclear whether some of these events are more "necessary and sufficient" than others in effecting the death of cells. A hint that this might be so is given by the observation that different members of the cascade appear to be preferentially selected in particular cell types and also show substrate specificity [12, 13].

The Bcl2 Family Members Define Thresholds for Apoptosis

The Bcl2 family members [2, 3, 14] are united through their possession of homologous domains responsible for protein–protein interactions amongst the family members. Bcl2 itself and its closest relatives (e.g. Bcl_{XL}) possess four such domains, BH 1, 2 and 4, defining a hydrophobic groove within the molecule, and BH3, a short (8–12 amino acid) region that binds within that groove. These members of the family support cell survival, whilst the shared BH domains permit interaction with two powerful pro-death molecules, Bax and Bak, through the formation of hetero-dimers. Bax and Bak (which possess the BH1-3 domains but not BH4) can also form homo-oligomers and, in this configuration, can create a wide diameter pore through cell membranes [15]. This event has been extensively studied in the mitochondrial membrane, where such pores allow escape of critical molecules from the intermembranous space to the immediate peri-mitochondrial microenvironment. Amongst the escaping molecules are cytochrome *c* and dATP, which together activate caspase 9, held in this microenvironment by its association with a protein (with remarkable, seven-fold symmetry) called apaf-1. The concentrations of Bax or Bak relative to the Bcl2-Bcl_{XL} or other pro-survival family members thus determine the probability that this dramatic rise in mitochondrial permeability will occur, activating the intrinsic pathway.

The remaining members of the Bcl2 family possess only the BH3 domain as their region of homology with the rest of this extended family. These proteins (bid, bad, bim, bmf and others) all promote death. Although their precise mode of action is still disputed, a likely explanation can be found in their high affinity of binding, via the BH3 domain, to the hydrophobic groove in Bcl2 and Bcl_{XL} [14, 16]. A rise in cell concentration of BH3-only proteins will therefore create conditions in the immediate vicinity of the mitochondrial outer membrane that favour formation of bax/bak oligomer formation and the genesis of the high-permeability pores. This widely diverse family of "BH3-only" proteins appears to provide signals in response to a variety of injuries (Fig. 1). Thus, they act as sensors of "danger" or "stress" conditions.

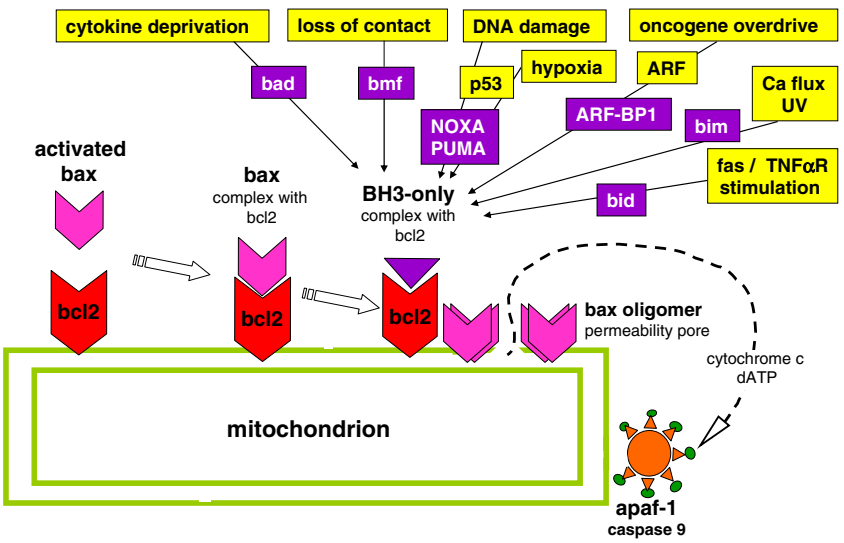

Fig. 1 Diagrammatic representation of the interaction of BH3-only members of the BCl2 family with the bax/bak–BCl2/BCl_{XL} complexes on mitochondrial membranes, relative to a variety of injury stimuli

Other Modes of Cell Death Exist

Apoptosis is widely observed in metazoans, but it is not the only route to cell death, even in the context of development. Moreover, even in organisms in which members of both the caspase and Bcl2 family are present constitutively, cells can sometimes undergo developmentally determined death despite experimental inhibition of caspases, showing that in these circumstances, the event of death must be determined by elements upstream of (or at least parallel to) the caspases themselves [17]. Interestingly, however, the structural changes in such dying cells differ from those of apoptosis and appear rather to represent loss of cellular volume homeostasis [18–20].

Despite the detail in which apoptosis is now understood, several major questions remain. Amongst these are two that are the subjects of the remainder of this short review. Both relate to apoptosis following cell injury. Under these circumstances, both in vivo and in vitro, it is usual to observe some cells entering apoptosis while their neighbours do not, despite being exposed to very similar lethal stimuli. The surviving cells often exhibit adaptive reactions that sustain cell life even in these unfavourable circumstances [21–23]. The questions therefore arise: first, what is the intracellular audit that determines which cells are selected for life, others for death? And second, what is the switch that determines that these adaptive changes are abandoned in favour of apoptosis? This article attempts to address both these questions, using cell damage by ionising radiation (IR) as the paradigm. The questions themselves, however, and hopefully their answers, are likely to be of general import.

The Search for an Intracellular Audit of DNA Damage

It is often assumed that apoptosis is initiated if the damage is "too severe to be repaired" or if the timescale for complete repair is "too long". Although these statements seem probable, there is little evidence for the mechanism responsible. There is, however, excellent evidence that DNA repair is initiated swiftly after DNA damage by IR in cells destined to die by apoptosis a few hours later and is nearly complete before apoptosis is initiated. Thus, for example, comet assays show nearly complete restoration of supercoiled DNA within 1 hour of irradiation of bone-marrow-derived pre-B cells, whilst apoptosis is not evident until some 2 to 4 hours later, even under conditions in which more than 90% of the cells eventually die [24]. This suggests either that some lesion other than DNA breakage itself is involved in signalling apoptosis, or (following the basic design of other checkpoints) the defining event is the repair of the last persisting double-strand break (DSB), and failed completion of this, within some critical "window" in time, or perhaps in some other intracellular condition, spells death.

We argued that if the audit of cell injury following IR is based on DNA damage, its location is likely to be close to the damage itself, i.e. in an intranuclear site. This immediately contrasts with current paradigms, which, as outlined earlier, place the signals for apoptosis either at the cell membrane or in the vicinity of mitochondria. To be credible, a candidate audit mechanism for DNA damage would be expected to be responsive in a dose-dependent manner to the intranuclear DNA damage itself. Moreover, the audit apparatus would be expected to demonstrate a dose-related qualitative transition when responding to lethal as opposed to survivable levels of damage.

One candidate with these properties has been described—the Promyelocytic Leukaemia Nuclear Domain (PML-ND) or PML body. PML bodies are intranuclear particles consisting of a shell of around 0.2-μm diameter, constituted of polymerised sumoylated PML protein, together with a wide array of cargo proteins [25]. Most cells in culture have around 10 PML bodies per nucleus, although the precise number in any one population is influenced by cell type and position in the cell cycle. Notably, however, the number of PML bodies per nucleus rises dramatically following DNA damage, in human fibroblasts often by some 200% to 300% [26–29]. This increase peaks around 4 to 8 hours after DNA damage, reverting to near normal values by 12 to 24 hours if the IR dose is low (≤2.5 Gy), but remaining high if the IR dose is high (≥5 Gy). Interestingly, this transition corresponds to the transition between sublethal and lethal doses at least in terms of "reproductive death" (i.e. irreversible replication arrest). Another striking feature of PML bodies is their intranuclear location relative to the foci at which DNA damage and repair take place (IR-induced foci, or IRIFs). Initially following radiation, there is no particular spatial relationship between PML bodies and IRIFs, but within a few hours, most PML bodies are closely adjacent to IRIFs [26, 29] (Fig. 2). These features suggest, but do not prove, that the surface of PML- bodies is capable of identifying the status of damaged DNA as it undergoes repair.

The mechanism underlying this alteration in number of the bodies is not known for certain, but is considered to be the remit of protein modification rather than transcription or translational events, since it is not affected by blockade of protein synthesis by, for example, cycloheximide. Some data suggest that larger bodies split or bud to create increased numbers of smaller ones [30]. This might influence their capacity to permit interaction of their cargo proteins, or perhaps might transiently increase the reactive surface available for such interactions. However, this interpretation does not exclude other possibilities, such as

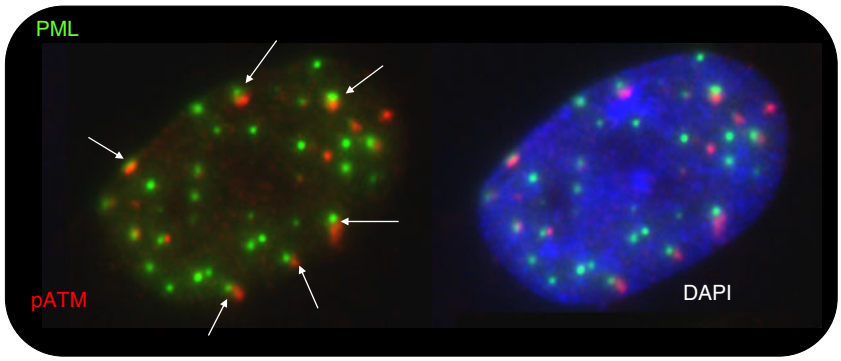

Fig. 2 Nucleus of a cell injured by IR 4 hours previously, showing the juxtaposition of IR induced foci (identified by an antibody to ATM, labelled *red*) with PML bodies (identified by an antibody to PML, labelled *green*). Courtesy of Dr. Brian Ferguson

PML-ND turnover, perhaps through differential sumoylation, in the vicinity of DSBs.

These observations are at least consistent with the hypothesis that PML bodies are a part of the nuclear response to DNA injury. Additional evidence comes from study of the nature of the cargo proteins, which include several involved in the response to injury, such as p53 (whose activation by acetylation and stabilisation by mdm2 appear to be facilitated by the presence of PML protein) [31–35], Blm (a helicase critical for completion of DSB repair) [36, 37] and Daxx (a modulator of apoptosis) [38]. Finally, and most significantly, tissues from animals genetically deficient in PML show attenuated apoptosis in response to a variety of lethal stimuli [39].

How might PML bodies become "aware" that the nucleus has sustained DNA damage?" The answer to this important question is not known, but a clue may be offered from consideration of the topographic molecular rearrangements that take place within the damaged nucleus. DSBs are swiftly reorganised by the DNA-dependent kinase ataxia telangiectasia mutated (ATM), which is phosphorylated close to the break site. Substrates of this kinase include many of the molecules that participate in DNA repair, such as the MRN complex, p53 BP1 and BRCA1 [40–42]. Detailed study of nuclear topology shows that these ATM substrates are activated in chromatin surrounding the break site, in association with the modified histone γH_2AX [43]. Phosphorylation of this probably leads to the unwinding of chromatin that facilitates access of the repair molecules to the break site. A further class of repair-associated molecule is typified by the checkpoint-related kinases Chk-1 and Chk-2, which move freely throughout the damaged nucleus and act both as substrate for and a stimulus to some of the other phosphorylation events involved in repair. Interestingly, Chk-2-deficient cells show a grossly retarded PML response to DNA injury, suggesting that Chk-2 may be part of the signal that informs PML of the intranuclear presence of DNA DSBs.

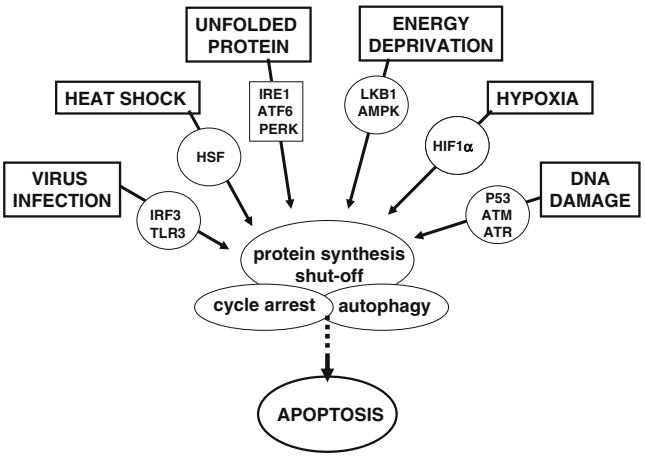

Fig. 3 A variety of different potentially lethal stimuli, each recognised by a specific set of transcription factors and signalling molecules, initiate the adaptive reactions of cycle arrest, autophagy and protein synthesis shutoff. For reasons that are still poorly understood, these reactions can be over-ruled by cell death, apparently by an endogenously controlled mechanism

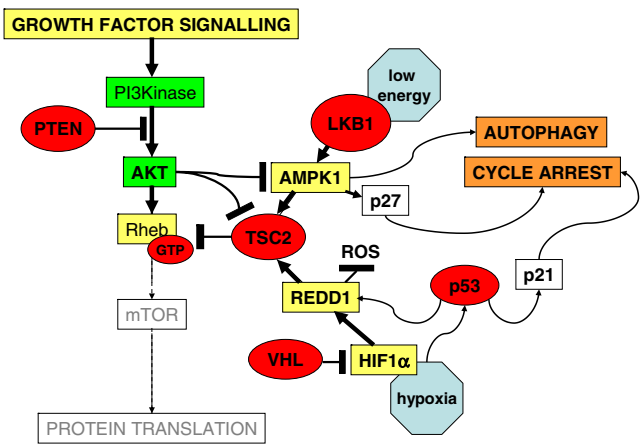

Fig. 4 Schematic diagram showing the relationship between AKT activation (survival stimulus) with adaptive reactions to oxygen and nutrient deprivation that lead to autophagy, cycle arrest and inhibition of the AKT/mTOR pathway that would otherwise have stimulated protein translation

The Switch From Adaptive Survival to Apoptosis

A still broader question asks what the relationship might be between the adaptive responses that support cell viability following injury and the homeostatic regulatory response that commits injured cells to death. The adaptive responses are heterogeneous, in that very different "stress" stimuli initiate stimulus-specific detection and immediate response mechanisms (Fig. 3). It is clear that, for example, quite distinctive intracellular networks are engaged in the reaction to endoplasmic reticulum stress (the unfolded protein response) [44–46], viral infection (including the interferon response) [47] and heat shock [48–50]. However, the ultimate cellular reactions appear to represent a common pathway, in which autophagy, protein synthesis shutoff and inhibition of DNA replication are conspicuous features. Although knowledge of the driving mechanism behind each of these fundamental adaptive responses to injury is still incomplete, a picture is emerging in which the PI3 kinase/Akt pathway plays a significant role in all three (Fig. 4). Interestingly, this pathway is also involved in the initiation of apoptosis and may include an intranuclear element that incorporates PML bodies in critical sites determining the outcome of Akt-driven phosphorylation [51].

One possible method to over-ride adaptive responses would be to recruit the innate immune system's killing power, through exposure of a "stress-dependent" or "danger" signal on the cell surface. One known example of this is the expression of the immunoglobulin-like molecules mic A and mic B on the surface of stressed cells [52]. These molecules (which are not expressed in this way in the absence of cellular stress) engage with NK cell receptors and permit activation of the latter's killing mechanism. It seems improbable, however, that a process as important as switching from survival to death should depend exclusively on a non-cell autonomous strategy such as NK cell activation.

Cell autonomous switches from adaptive responses to apoptosis in injured cells also exist. One example is the initiation of apoptosis following extremes of endoplasmic reticulum stress. Here, the transcription factor CHOP (for C/EBP homologous protein), a factor in the unfolded protein response, participates in the adaptation to protein overload, being transcriptionally activated in response to inducers of the unfolded protein response (ATF4, ATF6, XBP1) and also to ATF2, a molecular sensor for hypoxia and amino acid starvation. Over-expression of CHOP, however, initiates apoptosis [53, 54]. A mechanism of this type, in which signals emanating from within the stressed cell are both adaptive and pro-apoptotic, can be readily fitted into scenarios in which the permit to activate apoptosis is regulated through modification of the cell's apoptosis threshold—as described for BH3-only proteins or PML bodies above.

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